

An evaluation of the heat precipitation method for plasma fibrinogen estimation

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SYNOPSIS A rapid heat precipitation micromethod for the estimation of plasma fibrinogen is described. Its validity and reproducibility have been extensively tested. The method correlates well with a more generally used thrombin-clotting technique over a wide range of fibrinogen levels.

The methods available for the assay of plasma fibrinogen depend on its coagulability by thrombin, its behaviour on salt-fractionation, its antigenicity, its electrophoretic mobility, or its heat precipitability. They all contain important sources of possible error (Huseby and Bang, 1971). The most generally accepted techniques are based on the estimation of fibrinogen as fibrin after thrombin conversion; they are, however, time-consuming and unsuitable for rapid estimations on large numbers of samples. The method most easily adopted as a simple, rapid screening measure is the heat precipitation technique.

Fredericq in 1877 first demonstrated that a plasma component essential for clotting was precipitated at 56°C. Methods for the quantification of this heat-precipitated fraction, which is the plasma fibrinogen, have been described by Schulz (1955), Goodwin (1965), and Low, Hill, and Searcy (1967). The technique is not in general use presumably because of doubts as to its validity and reproducibility; these have been investigated in the present study by a rapid heat precipitation micromethod, based on that described by Low *et al* (1967).

Methods

THE HEAT PRECIPITATION TECHNIQUE

Venous blood is collected into sequestrene and drawn into two microhaematocrit capillary tubes. These are sealed at one end by heat and spun in a Hawksley microhaematocrit centrifuge (12,000 × *g*) for five minutes. The tubes are placed for three minutes in a waterbath at 56°C (± 1°C), care being taken to ensure that the plasma columns are entirely under the water surface. The plasma becomes opaque due to precipitation of the fibrinogen which is then packed on top of the buffy coat by centrifugation

for a further three minutes. The capillary tubes are placed on a movable microscope stage fitted with a vernier and viewed by transmitted light at a magnification of × 24. The focusing eyepiece contains a hair line. The length of the column of precipitate is measured in relation to that of the original plasma column (Fig. 1): readings are made to the nearest 0.1 mm on the vernier. The amount of fibrinogen in the plasma is then $AB/AC \times 100 = \text{ml}/100 \text{ ml}$. The mean of the readings in the two tubes is taken.

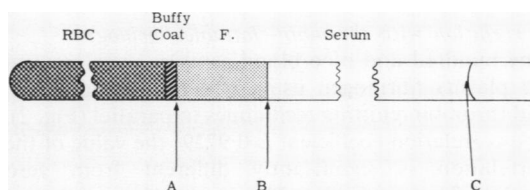


Fig. 1 Diagrammatic representation of the capillary tube contents after the heat precipitation and centrifugation of fibrinogen, showing packed fibrinogen F, buffy coat/fibrinogen interface A, fibrinogen/serum interface B and serum meniscus C.

THROMBIN-CLOTTABLE FIBRINOGEN

The method employed was that of Ogston and Ogston (1966), which is a modification of that of Ratnoff and Menzie (1951). The fibrin formed by the action of thrombin on fibrinogen is hydrolysed by boiling alkali and its tyrosine content measured with Folin-Ciocalteu's phenol reagent.

PREPARATION OF FIBRINOGEN DEGRADATION PRODUCTS (FDP)

Five hundred Ploug units of urokinase (Leo) were added to 25 ml of a 0.4% solution of human fibrinogen (Kabi). The mixture was incubated at 37°C for

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30 minutes at which time 1.9 ml of a 2000 KIU/ml solution of Trasylol (Bayer) was added to prevent further proteolysis. Undigested fibrinogen was clotted by thrombin and removed by centrifugation.

MEASUREMENT OF FIBRINOGEN DEGRADATION PRODUCTS

The method used was the tanned red cell haemagglutination inhibition (TRCHI) technique using the Wellcome kit. It was not possible to remove completely heterophile antibodies despite repeated absorption with sheep red cells: this method would thus be unreliable for the detection of low levels of fibrinogen degradation products.

IMMUNODIFFUSION

This was performed after the method of Mansi (1958) using rabbit antisera (Behringwerke) to a wide variety of human plasma proteins.

Results

VALIDITY OF THE METHOD

Addition of fibrinogen to saline, serum, and plasma

Known incremental amounts of fibrinogen were added to volumes of saline, serum, and plasma and the heat precipitation test was performed. In every instance the predicted fibrinogen level was observed.

Correlation with thrombin-clottable fibrinogen

One hundred and nine blood samples were assayed for plasma fibrinogen using the heat-precipitation and thrombin-clotting techniques in parallel (Fig. 2). The correlation coefficient is 0.9729: the value of the correlation is significantly different from zero ($t = 13.83$, $P < 0.001$). The regression equation of the heat precipitation method on the thrombin-clotting method is $x = 2.5771 + 0.9871(y - 3.1415)$, when x is the heat precipitate value for a given value, y , of the clotting method. It might have been expected that the regression line would pass through the origin ($x = y = 0$) but the intercept on the y axis is 0.53. This suggests that either the heat precipitation method underestimates or the clotting method overestimates the true value by a small fixed quantity. The graph could, of course, be used for the conversion of heat precipitate values to the more standard mg/100 ml unit.

A similar study on 23 rabbit blood samples again gave good correlation between the two methods although a statistical analysis has not been made.

Effect of FDP on the heat precipitation method

The FDP preparation had a high total concentration of 5,000 $\mu\text{g/ml}$ as tested by the TRCHI technique.

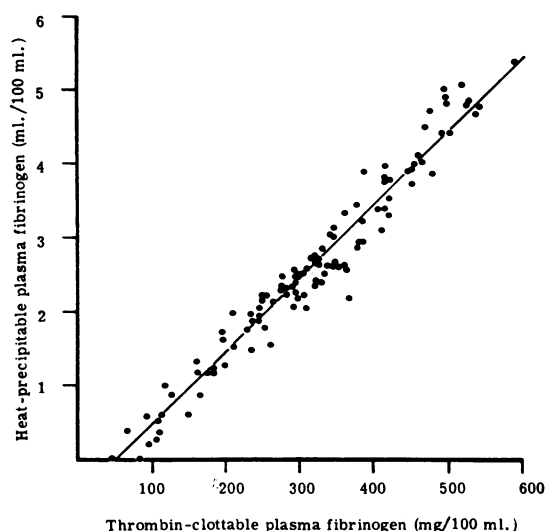


Fig. 2 Correlation between heat-precipitation and thrombin-clottable methods for fibrinogen estimation.

Eighty per cent of this FDP preparation was precipitable at 56°C, giving a value of 1 ml/100 ml: the supernatant comprised fragment E which is not precipitated at this temperature. Theoretically, to give a notably false reading, a concentration of at least 1,000 $\mu\text{g/ml}$ (0.2 ml/100 ml) total FDP would have to be present in the plasma under test. To test this, incremental amounts of FDP were added to normal plasma: only at levels of 1,000 $\mu\text{g/ml}$ or higher was there an increase in the resultant precipitate outside a 10% allowable limit. Fibrinogen degradation products did not prevent the heat precipitation of fibrinogen itself, although they may interfere with its conversion to fibrin in a thrombin-clotting technique (Hirsh, Fletcher, and Sherry, 1965).

Effect of heating on other plasma proteins

Immunodiffusion showed that no fibrinogen remained in the supernatant after heat precipitation and centrifugation. Electrophoresis showed that no other normal plasma protein was significantly altered, either quantitatively or electrically, by heating to this temperature.

REPRODUCIBILITY OF THE METHOD

Type of anticoagulant

Blood samples were collected in various standard anticoagulants, namely, sequestrene, ammonium potassium oxalate, sodium citrate, and heparin. No significant difference was noted in the fibrinogen values obtained. Sequestrene anticoagulation, how-

ever, was found to give the most distinct interface between the buffy coat and the heat precipitate and is regarded as the anticoagulant of choice.

Storage of blood

Storage of blood overnight at 4°C made no difference to the estimations. Freezing of plasma alone and storage at -20°C for up to one month similarly had no effect, although repeated freezing and thawing did produce inconsistent results.

Duration of heating and centrifugation

Five minutes' initial centrifugation, three minutes' heating, and three minutes' recentrifugation were found to be optimal. Shorter times gave inconsistent results, while prolongation of any step to 15 minutes did not alter the results.

Temperature

There was no variation in results within a temperature range of 55 to 57°C.

Observer variation and test-retest difference

A theoretical source of observer error is precise localization of the interfaces (lines A and B in Fig. 1). The buffy coat is pale brown compared with the white homogeneous layer of packed fibrinogen. Occasionally lines A and B are not strictly at right angles to the tube wall and the 'line of best fit' has to be chosen. If, as rarely happens, the interfaces are extremely irregular, the test is repeated. Precise vernier reading is essential.

From 70 blood samples two separate aliquots were taken and on each of these two fibrinogen estimations were made. The mean difference was 0.124 ml/100 ml (SD \pm 0.114). Ninety-three per cent of the estimations had a test-retest difference of less than 0.3 ml/100 ml. Any test showing a difference between the two tubes of more than 0.3 ml/100 ml should therefore be repeated: this difference is equivalent to 30 mg/100 ml, ie, about 10% in the normal plasma fibrinogen range.

Discussion

This heat precipitation method for plasma fibrinogen measurement requires little technical skill, apart from precise vernier reading, and no chemical reagents. The equipment used is simple and available in many siderooms: in the absence of a water bath, a simple tap-water bath is adequate. One sample can be assayed in 15 minutes, a dozen in half an hour. The method correlates well with a more generally used thrombin-clotting technique over a wide range of fibrinogen levels.

We have confirmed the results of Beller and Maki

(1967) that FDPs do not prevent the heat precipitation of fibrinogen. Certain FDPs are themselves heat precipitable and a false high value may be obtained if they are present in very high concentration. Some of these FDPs are also incorporated into the clot (Alkjaersig, Fletcher, and Sherry, 1962) so that the simultaneous heating of plasma and serum samples will not give an accurate estimation of the proportion of FDP in the plasma precipitate. As we have shown, however, FDP concentrations in plasma must exceed 1,000 μ g/ml before the reading is increased outside the limits of error of the method: such levels are rare, even in defibrination secondary to severe obstetric haemorrhage (Bonnar, Davidson, Pidgeon, McNicol, and Douglas, 1969).

Variable results have been noted in the heat precipitability of fibrinogen in jaundice (Foster, DeNatale, and Dotti, 1959) and rare abnormal plasma proteins may be precipitated at 56°C (Collier, Reich, and King, 1951) but will also be precipitated in heated serum.

The heat precipitation method seems particularly suitable as a screening technique. We have used it in a serial study of fibrinogen levels in over a thousand pregnant women, and in several instances where defibrination occurred the results correlated well with those obtained by the thrombin-clotting method.

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